

Evaluation of the Specificities of Five DNA Amplification Methods for the Detection of *Neisseria gonorrhoeae*

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The intragenus specificities of five molecular diagnostic methods for *Neisseria gonorrhoeae* were determined. Three assays were considered suboptimal. Molecular detection of *N. gonorrhoeae* from sites where other *Neisseria* spp. commonly occur or from any site in low-prevalence settings should be confirmed by a test targeting a different genetic locus.

The United Kingdom national guidelines for laboratory diagnosis of *Neisseria gonorrhoeae* recommend culture of the organism followed by confirmatory tests, which, for most specimens, is highly sensitive and specific. However, the sensitivity of culture may be suboptimal from specimen types such as pharyngeal and rectal swabs and synovial fluids (14, 21). Molecular diagnostic tests may be more appropriate for these sample types.

Intra- and interspecies genetic recombination occurs between members of the genus *Neisseria* and may do so with surprising frequency (6, 15). This poses a problem for the specificity of DNA-based diagnostic tests for *N. gonorrhoeae*, since the detection of a single gonococcus-specific gene that has been acquired by a commensal *Neisseria* isolate would result in a false-positive laboratory diagnosis. Assays of the highest specificity are required for testing extragenital specimens, in particular pharyngeal specimens, because this site commonly harbors commensal *Neisseria* and/or *Neisseria meningitidis* (11, 16).

Commercial molecular diagnostic tests for *N. gonorrhoeae* are designed for use on genital swabs and urine specimens, but some have also been evaluated for their use on extragenital specimens, such as pharyngeal and rectal swabs (17, 18, 21; H. Young, A. Moyes, and A. McMillan, Abstr. 12th Meet. Int. Soc. Sex. Transm. Dis. Res., abstr. O182, 1997). These studies suggest that, even with these specimen types, molecular methods can provide increased sensitivity compared to culture. However, molecular diagnostic assays for *N. gonorrhoeae* are usually evaluated during development with few or perhaps even single isolates of each species of *Neisseria*, so their true specificity within the genus remains unknown. This study evaluated the specificity of three commercial nucleic acid amplification assays and two published PCR assays by using isolates of *Neisseria* spp. that inhabit the human mucosal membranes.

A collection of 104 epidemiologically unrelated isolates from the genus *Neisseria* was used in this study. The collection consisted of the following: 24 *N. gonorrhoeae* isolates, including

20 of the proline-requiring, arginine-requiring (not satisfied by ornithine), uracil-requiring (PA^oU) auxotype, 10 *N. meningitidis* isolates, 23 *Neisseria cinerea* isolates, 6 *Neisseria flavescens* isolates, 11 *Neisseria subflava* isolates, 7 *Neisseria mucosa* isolates, 13 *Neisseria lactamica* isolates, 8 *Neisseria sicca* isolates, and 2 *Neisseria elongata* isolates. Of the nongonococcal isolates, 13 were National Collection of Type Cultures strains and the remaining 67 were originally isolated from a range of body sites: 15 ocular (13 of which were from infants under 1 year of age), 24 oral, 7 genital, 3 from blood, 1 each from abdominal, tracheal, wound, and urine samples, and 15 from unspecified sites. All isolates were grown on heated blood agar plates overnight at 37°C with 5% CO₂.

Bacteria were suspended in 100 µl of sterile distilled water to give a visibly turbid suspension and were mixed with 50 µl of 15% (wt/vol) Chelex-100 resin slurry (Bio-Rad Laboratories Ltd., Hemel Hempstead, United Kingdom). Samples were heated (95°C for 10 min) and centrifuged (2 min at 13,000 × g), and the supernatant was stored at –20°C.

The commercial assays were performed according to the manufacturers' instructions. The assays were a ligase chain reaction (LCR) targeting an *opa* gene (LCx; Abbott Laboratories), a PCR targeting the cytosine DNA methyltransferase gene (Amplicor; Roche), and a strand displacement amplification assay targeting DNA sequence homologous to a site-specific recombinase gene, Piv_{Ng} (2) (BDProbeTec amplified DNA assay; Becton Dickinson and Co.). Two published PCR assays, targeting the gene encoding outer membrane protein III (*ompIII*) (14) and the *cppB* gene (8), were performed as originally described. The Amplicor PCR was carried out using a PE Biosystems 9600 thermal cycler, and all other PCR assays were carried out using a PE Biosystems 9700 thermal cycler by using the 9600-ramp speed. Each assay was tested with the same amount of DNA (equivalent to 2 µl of lysates), and any nongonococcal isolate that was positive was repeated using a newly prepared DNA lysate.

Amplicons from the *ompIII* and *cppB* PCRs were analyzed by electrophoresis in a 2% agarose gel and were stained with ethidium bromide (10 µg/ml). *cppB* PCR products (10 µl) were digested for 1 h with 5 U of *MspI* (Gibco BRL) at 37°C in the appropriate buffer, and the products were analyzed as described above. For accurate determination of the size of am-

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TABLE 1. Comparative specificity of DNA amplification tests for *N. gonorrhoeae*

Test (source or reference)	Gene target	Data for false positives from 80 nongonococcal isolates ^a		Data for false negatives from 24 gonococcal isolates	
		Total	Identity	Total	Auxotype
LCx (Abbott)	<i>opa</i>	0		0	
PCR (14)	<i>ompIII</i> gene	0		0	
PCR (8)	<i>cppB</i>	1	<i>N. cinerea</i>	18	PA ^o U
PCR (Roche Amplicor)	Cytosine DNA methyltransferase gene	3	1 <i>N. flavescens</i> , 1 <i>N. lactamica</i> , 1 <i>N. sicca</i>	0	
SDA (BDProbeTec)	Piv _{Ng}	5	1 <i>N. flavescens</i> , 1 <i>N. lactamica</i> , 1 <i>N. subflava</i> , 2 <i>N. cinerea</i>	0	

^a All false-positive results were confirmed by repeating the test and by using a newly prepared DNA lysate.

plicons generated by the *ompIII* PCR, 10 µl of the product was analyzed using a 10% polyacrylamide gel (37.5:1 [wt/wt] acrylamide:bisacrylamide) in 1× Tris-borate-EDTA at 13.3 V/cm for 2.5 h. Acrylamide gels were stained for 15 min with 10 µg of ethidium bromide solution/ml, and all gels were photographed with UV transillumination using a Kodak DC120 digital camera.

The specificity of each assay is detailed in Table 1. With crude DNA preparations being used, the majority of nongonococcal isolates tested negative with most of the tests. The LCx and the *ompIII* PCR gave no false-positive results. However, from 10 commensal isolates the *ompIII* PCR produced amplicons of a size only slightly different (± 15 bp) from the expected 181-bp size of a true positive. This necessitated careful analysis of the PCR products with a 10% acrylamide gel. Reproducible false-positive results were obtained with five commensal isolates by using the BDProbeTec, with three isolates by using the Amplicor PCR, and with one isolate by using the *cppB* PCR. The *cppB* PCR also produced some nonspecific amplicons from nongonococcal isolates, but these were easily distinguishable by size from amplicons resulting from *N. gonorrhoeae* isolates, and the assay also includes a confirmatory restriction digest of the amplicon.

No single commensal isolate was positive by more than one test. Isolates that gave false-positive results were members of five different species: *N. cinerea*, *N. flavescens*, *N. lactamica*, *N. subflava*, and *N. sicca*.

The *cppB* PCR failed to detect 18 isolates of *N. gonorrhoeae*, all of which were of the PA^oU auxotype. The remaining four assays gave positive results with all 24 gonococcal isolates tested.

The specificity of DNA amplification-based detection methods for *N. gonorrhoeae* is problematic. One test, the *cppB* PCR, gave false-negative results, failing to detect 18 of 20 *N. gonorrhoeae* isolates of the PA^oU auxotype, a group that typically lacks plasmids (3). The *cppB* gene is found on the cryptic plasmid (13) but has been shown to integrate into the chromosome of some *N. gonorrhoeae* strains (7). A previous evaluation of the *cppB* PCR included only three plasmid-free strains, all of which were detected (20). This study, which used a greater number of isolates from the PA^oU auxotype, demonstrated that the *cppB* gene sequences targeted in this assay are not universally present in isolates of *N. gonorrhoeae*. Hybridization studies (9) have demonstrated homology between the cryptic plasmid and plasmids from *N. meningitidis* and *N.*

lactamica. This also raises concerns regarding false-positive detection of *N. gonorrhoeae* with the *cppB* PCR, particularly in pharyngeal swab specimens.

False-positive results with isolates of both *N. cinerea* and *N. subflava* have been reported previously for the Amplicor PCR (1996 Amplicor *Chlamydia trachomatis*/*N. gonorrhoeae* [CT/NG] test package insert, Roche Diagnostic Systems, Inc., Branchburg, N.J.) (4). This study demonstrates that not only the Amplicor PCR but also the BDProbeTec DNA amplification assay and the *cppB* PCR can give false-positive results from several different species of *Neisseria* (*N. cinerea*, *N. flavescens*, *N. lactamica*, *N. subflava*, and *N. sicca*). In this study crude DNA preparations were used that could contain quantities of DNA in excess of that encountered in clinical samples and might, therefore, be considered an unrealistic test of the assays. However, the validity of this approach is supported by the observations that the majority of isolates gave negative results with most of the assays and that no single isolate gave a false-positive result with more than one test. Obtaining positive results from nongonococcal isolates in only one of five assays also ruled out the possibility of laboratory contamination of those samples with gonococcal DNA.

These sporadic false positives are most likely to be the result of horizontal genetic exchange that occurs between members of the genus *Neisseria*, which coexist at the pharyngeal or genital mucosa. False-positive diagnoses of gonorrhea may occur, particularly from pharyngeal swab specimens, since human commensal *Neisseria* species and *N. meningitidis* are commonly isolated from the pharyngeal mucosa (11, 16). Nongonococcal *Neisseria* species (predominantly *N. meningitidis*) may be isolated infrequently from genital and rectal specimens (5, 10, 22). One study reported that these accounted for 0.38 and 0.66% of gram-negative diplococci isolated from male and female genital sites in heterosexuals and for 11.4% of gram-negative diplococci in rectal specimens from homosexual men (22). Additionally, there are documented cases where commensal *Neisseria* (*N. lactamica*, *N. cinerea*, *N. sicca*, and *N. subflava*) have been recovered from genital mucosa (1, 11, 12, 19), and in this study seven isolates (two *N. lactamica*, two *N. subflava*, and three *N. cinerea* isolates) were originally isolated from genital sites. Hence, the identification of false positives from genital specimens is also possible.

Two assays (the PCR targeting the *ompIII* gene and the LCx targeting an *opa* gene) gave no false-positive or false-negative results. Possibly, successful genetic exchange at these loci oc-

curs less frequently than at other loci, making these good targets for use in diagnostic tests. However, unless specific loci are protected from genetic recombination events, then false-positive results attributable to nongonococcal *Neisseria* sp. will, in time, be identified for every test.

Molecular diagnoses that are based on a single test risk false-positive results. This is of greatest concern when pharyngeal specimens are tested but is also relevant for genital specimens, particularly in low-prevalence settings. One way to avoid false-positive results is to carry out a confirmatory test that targets a separate region of the genome, since it is unlikely that a single organism will recombine two *N. gonorrhoeae* specific genes that are located separately.

Recent evaluations of amplified DNA detection tests on specimens from extragenital sites have used the LCR assay (17, 18; Young et al., 12th Meet. Int. Soc. Sex. Transm. Dis. Res.). These studies all demonstrated significant increases in detection of pharyngeal gonorrhea compared with results of detection by culture. In all three studies, the LCR assay targeting an *opa* gene was confirmed with a second LCR assay targeting a pilin gene (the latter is presently only made available by the manufacturer upon request) and specimens were only considered positive if both targets gave positive results. The two larger studies (17; Young et al., 12th Meet. Int. Soc. Sex. Transm. Dis. Res.), analyzing 200 and 330 samples, identified two to four isolates that were not confirmed by the pilin LCR. It is possible that some or all of these "LCR *opa* positive-LCR pilin negative" isolates may result from the detection of commensal *Neisseria* that has acquired a gonococcal *opa* gene.

It is established that DNA amplification assays offer increased sensitivity compared with culture, which in turn could improve detection of gonorrhea in extragenital sites where infection may be asymptomatic and hence otherwise go untreated. However, given the promiscuous interspecific genetic recombination that occurs within the genus *Neisseria*, we believe that molecular detection of gonorrhea in extragenital specimens and those from low-prevalence populations should be subject to a confirmatory test that targets a separate region of the genome.

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